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Determination of Nitrogen-Fixing Phylotypes in *Lyngbya* sp. and *Microcoleus chthonoplastes* Cyanobacterial Mats from Guerrero Negro, Baja California, Mexico

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In many environments, biological nitrogen fixation can alleviate nitrogen limitation. The high rates of N_2 fixation often observed in cyanobacterial mats suggest that N_2 fixation may be an important source of N. In this study, organisms expressing *nifH* were identified in a *Lyngbya* sp.- and two *Microcoleus chthonoplastes*-dominated cyanobacterial mats. The pattern of nitrogenase activity was determined for the *Lyngbya* sp. mat and a *Microcoleus chthonoplastes* mat sampled directly in Guerrero Negro, Mexico. Their maximum rates were 23 and 15 μmol of C_2H_4 m^{-2} h^{-1}, respectively. The second *Microcoleus* mat, which was maintained in a greenhouse facility, had a maximum rate of 40 μmol of C_2H_4 m^{-2} h^{-1}. The overall diel pattern of nitrogenase activity in the three mats was similar, with the highest rates of activity occurring during the dark period. Analysis of *nifH* transcripts by reverse transcription-PCR revealed that several different organisms were expressing *nifH* during the dark period. *nifH* phylotypes recovered from these mats were similar to sequences from the unicellular cyanobacterial genera *Halothece*, *Myxosarcina*, and *Synechocystis,* the filamentous cyanobacterial genera *Plectonema* and *Phormidium*, and several bacterial *nifH* groups. The results of this study indicate that several different organisms, some of which were not previously known to fix nitrogen, are likely to be responsible for the observed dark-period nitrogenase activity in these cyanobacterial mats.

Microbial mats are benthic laminated microbial assemblages that are persistent biological features in many environments. They are found in many different aquatic habitats, including hydrothermal vents, hot springs, ponds, and lakes (20, 22, 24, 25). Cyanobacterial mats are microbial mats that contain cyanobacteria but typically host a large diversity of microorganisms that function in numerous microbially mediated processes. Microbial processes in mats include photosynthesis, sulfate reduction, methanogenesis, and nitrogen fixation (5, 7, 16). Thus, there are strong light and chemical gradients in mats that both control and result from the vertical stratification of microorganisms and biochemical activities in the mats.

In many environments, nitrogen availability can limit growth and ecosystem productivity (44). Biological nitrogen fixation, or diazotrophy, the fixation of atmospheric nitrogen gas (N_2) into biologically available ammonia (NH_3), is important in making nitrogen available in many ecosystems, including microbial mats (18, 27, 44). The ability to fix N_2 is widely distributed among diverse members of the *Bacteria* and *Archaea* (46). The nitrogenase protein which catalyzes the conversion of N_2 to NH_3 is highly conserved among microorganisms and is likely to have evolved early (33, 46). Nitrogen fixation is inhibited by oxygen due to the sensitivity of nitrogenase to oxygen inactivation (17). Oxygen-evolving cyanobacteria have developed various strategies, such as temporal or spatial segregation of N_2 fixation (into heterocysts) and oxygen evolution, to avoid oxygen inactivation (14). Cyanobacterial mats contain diverse types of microorganisms that could potentially fix N_2 but also exhibit high concentrations of oxygen due to oxygenation from the atmosphere and cyanobacterial photosynthesis (2, 12). Thus, it is often unclear which microorganisms may be involved in N_2 fixation and how N_2 fixation in mats proceeds in the presence of oxygen-evolving phototrophs.

Cyanobacterial mats, which contain diverse cyanobacterial taxa, including heterocystous cyanobacteria, filamentous nonheterocystous cyanobacteria, and unicellular cyanobacteria, often exhibit high rates of nitrogen fixation (4, 5, 13, 28, 30). The temporal patterns of N_2 fixation observed in cyanobacterial mats are often similar to those observed in cultures of cyanobacteria (35), suggesting that cyanobacteria may play a role in mat N_2 fixation. However, N_2 fixation may also be driven by the activities of anoxygenic photoautotrophs, including green and purple bacteria (31, 45). It is also possible that heterotrophic bacteria are involved in N_2 fixation, fueled by photosynthate released from phototrophs (27, 29).

Most studies of the organisms involved in N_2 fixation in cyanobacterial mats have focused on physiological and genetic characterizations (4, 26, 31, 36, 37, 48). Incubating microbial mats with the photosystem II inhibitor DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] and/or keeping mats in the dark during the daytime typically causes substantial reductions in nitrogenase activity during the subsequent night (4, 31). The decreased dark-period N_2 fixation is most likely due to a reduction in photosynthetic products which would fuel N_2 fixation.

Diversity studies in cyanobacterial mats have focused on

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amplifying and sequencing the *nifH* gene, one of the genes responsible for nitrogen fixation (26, 37, 48). These studies have shown that there is a diverse array of N_2 fixers that could be involved in N_2 fixation, including cyanobacteria, alpha-proteobacteria, gamma-proteobacteria, and delta-proteobacteria. One study attempted to identify which microorganisms actively participate in N_2 fixation (36). However, there is relatively little information on how the diversity of organisms involved in N_2 fixation differs between mats.

The purpose of this study was to determine the types of microorganisms involved in N_2 fixation in two microbial mats exhibiting very different rates of nitrogenase activity, the well-developed *Lyngbya* sp. and *Microcoleus chthonoplastes* mats, in Guerrero Negro, Baja California, Mexico. Previous work by Omoregie et al. (26) found numerous *nifH* sequences belonging to various members of the proteobacterial lineage as well as cyanobacteria and several unidentified bacteria. However, from that study it was unclear which of these organisms were active in N_2 fixation. We examined the rates of nitrogenase activity by acetylene reduction in relation to the expression of specific *nifH* gene phylotypes by reverse transcription-PCR. This method has already been proven effective in several studies (23, 36, 51). Our results show that unicellular and filamentous cyanobacteria as well as heterotrophic bacteria were expressing the nitrogenase gene during the dark period.

MATERIALS AND METHODS

Sampling. A *Lyngbya* sp.-dominated microbial mat (LG), freshly collected (GNM), and greenhouse-maintained *Microcoleus chthonoplastes* (GHM)-dominated mats were studied. The mats used in this study were sampled from the Exportadora de Sal salt works in Guerrero Negro, Baja California, Mexico. The Exportadora de Sal salt works is located approximately 720 km south of the United States border with Mexico.

Microcoleus chthonoplastes mats were collected from a hypersaline pond (area 4; salinity, ca. 90 to 78‰) in June 2001 (GHM) and October 2001 (GNM). Core samples of GNM mats were obtained immediately upon collection of mats from area 4. The GHM mats were maintained in a greenhouse facility at NASA Ames Research Center in Mountain View, Calif. (3), for approximately 1 month prior to sampling for this study in July 2001. The GHM samples were obtained from the same cores used in the study by Omoregie et al. (26).

Lyngbya (LG) mats were collected from a desiccated intertidal flat in October 2001. Because the tidal flat from which the *Lyngbya* mats were collected is flooded at irregular intervals by water from the Ojo de Liebre lagoon, samples of the mat were collected in plastic trays (20 by 25 cm) and transported to a nearby laboratory for time course (diel) experiments. At the field laboratory, the trays were filled with lagoon water from the nearby Ojo De Liebre (salinity, ca. 40‰) and incubated at in situ irradiance and temperature. LG mats were sampled from these trays in all experiments. Circular cores approximately 15 mm by 15 mm in dimension were taken from all three mats. Cores were sectioned at approximately 3- to 5-mm intervals and placed in cryovials, which were immediately frozen in liquid nitrogen and transferred to a −80°C freezer. An additional set of replicate cores ($n = 3$) were placed in serum bottles at each time point and used for acetylene reduction rate measurements (incubation period, 3 to 4 h). The top 3- to 5-mm section of each mat core was used for this study.

Acetylene reduction assay. Nitrogenase activity was measured by the acetylene reduction assay (38) following the procedure of Bebout et al. (4). Cores (ca. 15 mm by 5 mm) were placed in 38-ml serum bottles containing 20 ml of water (overlying water from each mat). Three replicate cores were taken from each mat during each sampling period. The bottles were sealed with rubber stoppers, and 2 ml of headspace was removed and replaced with 5 ml of acetylene. The bottles were returned to the flumes, trays, or ponds from which they were sampled to maintain the same light and temperature conditions as the mats from which they were sampled. After 3 to 4 h, incubations were terminated by shaking each bottle for 10 s. Two milliliters of headspace was injected into a 9.1-ml serum bottle filled with a 3.4 M solution of sodium chloride, displacing the salt solution through a vent needle, for short-term storage. Ethylene concentrations were quantified (usually within hours of sample collection) by injecting 0.1 ml of the headspace into a Shimadzu GC14A or GCMini2 gas chromatograph (Shimadzu, Kyoto, Japan).

Isolation of total RNA. Total RNA was extracted from mat samples by modification of the Tillett and Neilan method (42). Preheated xanthogenate-sodium dodecyl sulfate buffers (1.2 ml) was added to 25 to 40 mg of mat in a 2-ml Bead Beat tube. The tubes were agitated in a Fast Prep machine (Bio 101, Carlsbad, Calif.) for 1.5 min at speed setting 6. Two hundred microliters of chloroform-isoamyl alcohol was added to each tube, vortexed briefly, and then centrifuged for 5 min. The aqueous phase was added to 500 μl of phenol-chloroform-isoamyl alcohol. Extractions were repeated until no visible material was left at the interface. The aqueous phase was removed to a microcentrifuge tube, to which 1 volume of isopropanol added, and then placed on ice for 15 min. The tubes were centrifuged for 10 min. The isopropanol was aspirated, and the tubes were washed twice in 70% ethanol. RNA pellets were then resuspended in 50 μl of nuclease-free water (Ambion). Residual DNA was removed by two rounds of DNase (Ambion) treatment, followed by purification with the RNEasy mini kit (Qiagen, Hilden, Germany) and eluted in 50 μl of RNA storage solution. To demonstrate that the RNA was not contaminated with genomic DNA, 1 μl of RNase (Ambion) was added to aliquots of extracted RNA prior to DNase treatment.

Reverse transcription and amplification. Reverse transcription and amplification were carried out by a nested PCR approach with primers NIFH4 (5′-TTYTAYGGNAARGGNGG-3′, positions 564 to 562 in *A. vinelandii* M11579) and NIFH3 (5′-ATRTTRTTNGCNGCRTA-3′, positions 1102 to 1118 in *A. vinelandii* M11579) for the first round and NIFH1 (5′-TGYGAYCCNAARGCNGA-3′, positions 639 to 655 in *A. vinelandii* M11579) and NIFH2 (5′-ADNGCCATCATYTCNCC-3′ positions 984 to 1000 in *A. vinelandii* M11579) for the second round (N = A, G, C, or T; D = A, G, or T; Y = C or T; R = A or G) (50). Primers were synthesized and purified by polyacrylamide gel electrophoresis at New England Biolabs.

Two microliters of sample was added to a 50-μl Access RT-PCR (Promega) reaction. Each reaction contained 1× reaction buffer, 1 mM $MgSO_4$, 0.8 mM total deoxynucleoside triphosphates, 1 μM each NIFH3 and NIFH4, 5 U of *Tfl* DNA polymerase, and 5 U of avian myeloblastosis virus reverse transcriptase. Reaction master mixes containing all reagents excluding avian myeloblastosis virus reverse transcriptase were filtered through a 100-kDa filter (Millipore). After filtration, avian myeloblastosis virus reverse transcriptase was added to the master mix and aliquoted, and the template was added to each reaction mixture. Alternatively, reaction mixtures without avian myeloblastosis virus or *Tfl* reverse transcriptase were filtered through a 30-kDa filter (Millipore), and enzymes were added and then aliquoted. To demonstrate that reverse transcriptase was necessary for sample amplification and that contaminating genomic DNA was not present, control reactions without reverse transcriptase were also performed. Samples were reverse transcribed and amplified with the following cycling conditions: 48°C for 45 min and 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min, followed by 72°C for 7 min.

A second round of nested amplification was performed with the NIFH1 and NIFH2 primers. The final concentration in each 50-μl reaction mixture was 4 mM $MgCl_2$, 1× PCR buffer, 0.8 mM total deoxynucleoside triphosphates, 1 μM each forward primer and reverse primer, and 2.5 U of *Taq* (Promega). Core mixes were filtered as described above. The following cycling conditions were used for the second round: 94°C for 1 min, followed by 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min, and a final 72°C for 7 min.

Cloning and plasmid isolation. Amplification products were ligated and transformed with the pGEM-T vector kit (Promega). Transformation reactions were plated on Luria-Bertani (LB)-agar plates containing 100 μg ml^{-1} ampicillin, 0.5 mM isopropylthiogalactopyranoside (IPTG), and 80 μg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Individual colonies were used to inoculate overnight cultures containing 3 ml of LB broth and 100 μg ml^{-1} ampicillin. Plasmids from cell cultures were purified with the QIAprep Spin miniprep kit (Qiagen, Hilden, Germany).

DNA sequencing and analysis. Purified plasmids were sequenced in one direction with either the Sp6 or T7 primer (IDT) with the ABI Prism BigDye Terminator version 3.0 cycle sequencing kit (Applied Biosystems). Samples were sequenced on an ABI Prism 310 genetic analyzer (Applied Biosystems). Sequences were edited with the GCG program (Accelrys). Probabilistic alignments for all sequences and sequences from GenBank were generated from predicted amino acid sequences with the HMMR program in GCG. Percent identities were calculated with the distance program in GCG with Kimura correction; the calculated distances were then normalized to 100. Representative sequences were chosen and sequenced in the reverse direction with T7 or Sp6 primer. Phyloge-

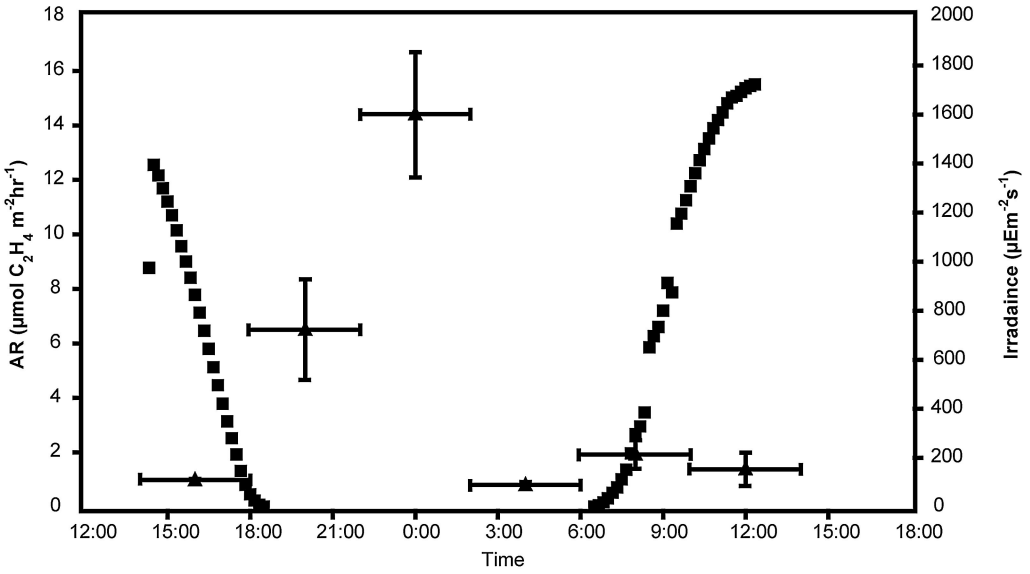

FIG. 1. Acetylene reduction (AR) rate measurements and light intensity for the October 2001 *Microcoleus* (GNM) mat. Solid squares (■) represent irradiance, and solid triangles (▲) represent the rate of acetylene reduction. Horizontal bars indicate the length of incubation. Vertical bars represent ±1 standard deviation of the average for three replicates. The highest rates of N_2 fixation (15 μmol of C_2H_4 m^{-2} h^{-1}) were measured between 22:00 and 2:00 h during the dark period.

netic distances were calculated from the probabilistic alignments with the distance correction algorithm of Tajima-Nei, available in the program TREECON for Windows (39, 43). Phylogenetic reconstructions from distance approximations were done by the neighbor-joining method in TREECON (34). A total of 100 bootstrap replicates were performed for each tree. The cluster 1 tree was rooted with a *nifH* gene from *Desulfovibrio vulgaris*, and the cluster 2, 3, and 4 trees were rooted with a *nifH*-like gene from *Plectonema boryanum*.

RESULTS

Nitrogenase activity. Nitrogenase activity for both mats (Fig. 1 and 2) was maximal at night and minimal during the day. This pattern of activity was inversely correlated to light intensity. The maximum rates of acetylene reduction for the LG and the

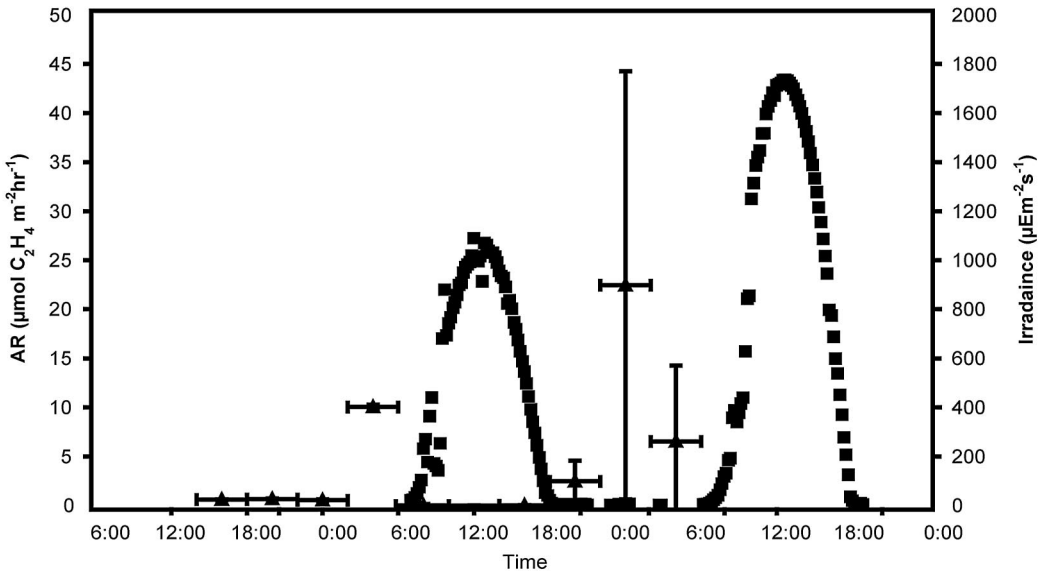

FIG. 2. Acetylene reduction (AR) rate measurements and light intensity for the *Lyngbya* mat (LG). Measurements were made over a day and half of sampling (39 h). Solid squares (■) represent irradiance, and solid triangles (▲) represent the rate of acetylene reduction. Horizontal bars indicate the length of incubation. Vertical bars represent ±1 standard deviation from the average for three replicates. The highest rates of N_2 fixation (23 μmol of C_2H_4 m^{-2} h^{-1}) were measured between 22:00 and 2:00 h (second day of measurements) during the dark period.

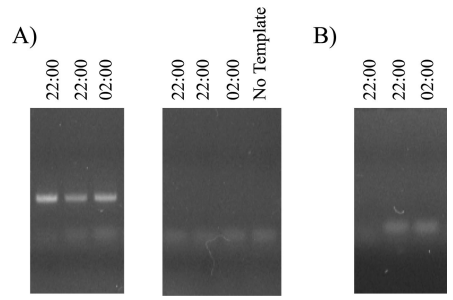

FIG. 3. Agarose gel showing reverse transcription and amplification of nighttime *nifH* mRNA from the *Lyngbya* mat (LG). Samples were collected at 22:00 h during the first day of incubation and at 22:00 and 2:00 h during the second day of incubation. (A) mRNA samples treated with DNase, and DNased samples treated with RNase prior to purification of mRNA, as well as a no-template control. (B) DNase samples to which no reverse transcriptase was added.

GNM mats were 23 and 15 μmol of C_2H_4 m^{-2} h^{-1}, respectively.

***nifH* gene transcription.** Since acetylene reduction rates were maximal at night, RNA samples collected at night were assayed for *nifH* expression. *nifH* transcripts were detected in all three mats during the dark. Figure 3 shows the product of an RT-PCR amplification of *nifH* mRNA from the LG mat. RT-PCR amplifications from the GHM and GNM mats (gels not shown) were similar to that of the LG mat. Samples taken from the LG and GHM mats at 22:00 and 2:00 h were positive for amplification of *nifH*. Additionally, samples taken from the GHM mat and the GNM mat at 18:00 h and from the GNM mat at 20:00 h were also positive for amplification of *nifH*. Samples to which RNase was added just prior to purification and samples in which reverse transcriptase was not included in the reaction mixture were all negative for amplification. In addition, no-template controls for all reactions were negative for amplification.

***nifH* sequence analysis.** A total of 110 cDNA sequences were recovered by RT-PCR in this study (Table 1). There were 67 sequences from the GHM mat, eight sequences from the GNM mat, and 35 sequences from the LG mat. In general, there are four major phylogenetic clusters of *nifH* protein sequences (9). Clusters 1 and 3 contain conventional (Mo-containing) nitrogenases from various proteobacteria, cyanobacteria, and firmicutes (cluster 1) and from clostridia, sulfate reducers, methanogens, and green sulfur bacteria (cluster 3). Cluster 2 contains "second alternative" (non-Mo- and non-V-containing) nitrogenases, and cluster 4 contains nitrogenase homologs. The majority of predicted *nifH* protein sequences obtained from the mats were in cluster 1 or 3 (Fig. 4 and 5). The sequence type GN2084A04, which was recovered from the LG mat, was deeply divergent and fell between clusters 2 (alternative nitrogenases) and 4 (*nifH* homologs). The closest isolate sequence type to this sequence in amino acid identity was that of *Desulfovibrio vulgaris* (70% identity).

A total of 91 sequences from this study were in cluster I of the *nifH* protein phylogeny. There were 63 amino acid sequences from all three microbial mats that ranged from 93 to 100% identity to the *nifH* protein from unicellular cyanobacteria, including *Halothece* sp. strain MPI96P605, *Myxosarcina* sp. strain ATCC 29377, and *Synechocystis* sp. strain WH8501. A total of 19 sequences from all three mats were 95 to 100% identical to the *nifH* protein from the filamentous cyanobacteria *Plectonema boryanum* and *Phormidium* sp. strain ATCC 29409. Five sequences from the GNM and LG mats were 95 to 97% identical to a second copy of *nifH* protein in the filamentous heterocystous cyanobacterium *Anabaena variabilis* ATCC 29413. Four sequences from the GHM mat were 86 to 88% identical to the *nifH* protein from the gamma-proteobacterium *Azotobacter vinelandii*.

nifH protein sequences in cluster 3 (Table 1 and Fig. 5) made up about 16% (18 sequences) of all the sequences recovered in this study. There were 3, 4, and 12 *nifH* protein sequences recovered from the GHM, GNM, and LG mats, respectively. This cluster of *nifH* sequences includes several lineages of anaerobic bacteria, such as clostridia, sulfate reducers, methanogens, and green sulfur bacteria. None of the *nifH* sequences in this cluster were closely related (88% or less identity) to sequences from bacterial isolates. Most of the cluster 3 sequences were more closely related (91 to 100%) to sequences recovered from other mats or ecosystems with anaerobic environments. The sequence types from cultivated isolates that were closest to any of the other mat cluster 3 sequences were those from delta-proteobacteria of the genus *Desulfovibrio*.

Of the 26 individual sequence types detected in this study, 12 were detected in DNA clone libraries (Table 1) in a previous study of these mats by Omoregie et al. (26). Two of the five sequence types recovered from the GHM mat were not detected by PCR in the same mat (26). Four of the seven RT-PCR sequence types from the GNM mat were detected by PCR (26). Furthermore, only one of these four sequence types from the GNM mat was recovered from the same mat (26). Five of the 13 sequence types from the LG mat were recovered by PCR of DNA (26). Only four of these sequence types were recovered from the same mat.

DISCUSSION

The diel pattern of nitrogenase activity (Fig. 1 and 2) for the *Lyngbya* (LG) mat and the *Microcoleus* (GNM) mat was cyclic, with the highest rates during the night and the lowest rates during the day. The pattern of acetylene reduction for the GHM mat was similar to the observed pattern in the LG and GNM mats (26). This pattern is consistent with the temporal separation of photosynthesis and N_2 fixation that has been observed in several filamentous nonheterocystous cyanobacterial mats and in cyanobacterial cultures (5, 6, 10, 21, 26). This pattern is typical for cyanobacteria which do not have heterocysts, the specialized cells in which nitrogen fixation is spatially separated from oxygenic photosynthesis in the vegetative cells (6). In filamentous nonheterocystous cyanobacteria, photosynthesis and nitrogen fixation are usually temporally separated, with photosynthesis occurring during the daytime and N_2 fixation occurring during the nighttime (14). Nitrogenase activity at night was anticipated because filamentous nonheterocystous cyanobacterial species are abundant in these mats.

The GNM mat had the lowest rates of acetylene reduction of the three mats studied. Low acetylene reduction rates are characteristic of this particular cyanobacterial mat (5; B. M.

TABLE 1. Summary of representative sequences recovered from the greenhouse *Microcoleus* (GHM), the October 2001 *Lyngbya* (LG), and the *Microcoleus* (GNM) mats[a]

Mat	Representative sequence	No. of clones	Closest isolate	% Identity[b]	% Identity of all sequences[c]	Phylogenetic affiliation	Closest sequence recovered previously[d]	Mat type[e]	% Identity	% Identity of all sequences
GHM	GN803A18	3	*Azotobacter vinelandii* M20568	88	86–88	Gamma proteobacteria	GN1064A02	GNM*	93	91–93
	GN803A10	7	*Plectonema boryanum* IU 594 L15552	98		Filamentous cyanobacteria	GN821A16	GHM	100	98–100
	GN812A20	0	*Halothece* sp. strain MP196P605	95		Unicellular cyanobacteria	GN821A05	GHM	96	
	GN821A45	50	*Halothece* sp. strain MP196P605	98	96–100	Unicellular cyanobacteria	GN821A05	GHM	99	97–100
	GN812A08	2	*Desulfovibrio salexigens* AF227926	84	83–84	Delta proteobacteria	GN821A01	GHM	100	99–100
GNM	GN2008A01	0	*Anabaena variabilis* ATCC 29413 U49859 (second copy)	96		Heterocystous cyanobacteria	GN1064A17	GNM*	99	
	GN2008A2	0	*Phormidium* sp. strain ATCC 29409 U73132	99		Filamentous cyanobacteria	GN1063A12	LG*	99	
	GN2013A02	0	*Halothece* sp. strain MP196P605	97		Unicellular cyanobacteria	GN821A05	GHM	96	
	GN2013A07	1	*Halothece* sp. strain MP196P605	98	97–98	Unicellular cyanobacteria	GN821A05	GHM	99	98–99
	GN2008A03	0	*Desulfovibrio salexigens* AF227926	84		Delta proteobacteria	GN821A01	GHM	93	
	GN2008A04	0	*Desulfovibrio vulgaris* AY040514	87		Delta proteobacteria	GN1063A36	LG*	99	
	GN2008A07	0	*Desulfovibrio salexigens* AF227926	83		Delta proteobacteria	GN821A01	GHM	92	
LG	GN2084A08	0	*Myxosarcina* sp. strain ATCC 29377 U73133	93		Unicellular cyanobacteria	GN821A05	GHM	90	
	GN2084A19	0	*Myxosarcina* sp. strain ATCC 29377 U73133	97		Unicellular cyanobacteria	GN821A05	GHM	94	
	GN2127A02	2	*Phormidium* sp. strain ATCC 29409 U73132	100	98–100	Filamentous cyanobacteria	GN1063A12	LG*	98	
	GN2127A09	1	*Myxosarcina* sp. strain ATCC 29377 U73133	96	95–96	Unicellular cyanobacteria	GN1062A41	LG	93	92–93
	GN2133A11	6	*Plectonema boryanum* IU 594 L15552	98	96–98	Filamentous cyanobacteria	GN821A16	GHM	100	98–100
	GN2133A17	3	*Synechocystis* sp. strain WH 8501 AF300829	94	93–94	Unicellular cyanobacteria	GN1063A15	LG*	100	98–100
	GN2133A02	3	*Anabaena variabilis* ATCC 29413 U49859 (second copy)	97	95–97	Heterocystous cyanobacteria	GN1063A08	LG*	98	96–98
	GN2084A04	0	*Desulfovibrio vulgaris* AY040514	70		Delta proteobacteria	GN1062A21	LG*	91	
	GN2084A12	0	*Desulfovibrio africanus* AY040512	86		Delta proteobacteria	GN1061A02	LG	92	
	GN2084A16	4	*Desulfovibrio salexigens* AF227926	89	88–89	Delta proteobacteria	GN1063A24	LG*	100	99–100
	GN2084A20	0	*Desulfovibrio salexigens* AF227926	84		Delta proteobacteria	GN821A01	GHM	87	
	GN2127A01	0	*Desulfovibrio salexigens* AF227926	86		Delta proteobacteria	GN821A15	GHM	90	
	GN2127A05	3	*Desulfovibrio salexigens* AF227926	86	84–85	Delta proteobacteria	GN821A01	GHM	93	91–93

[a] The number of sequences of each type is shown as well as their identity to the closest isolate sequence and to closest sequence obtained from DNA (26). Representative sequences represent sequences with greater or equal to 98% deduced amino acid sequence identity.
[b] Percent identity between the representative sequences and closest isolates.
[c] Percent identity between all sequences, including the representative sequence, to the closest isolate.
[d] Closest sequence recovered previously (26).
[e] *, sequence was derived from a developing *Microcoleus* or *Lyngbya* mat (26).


GN821A05 AY232400 ♣
GN1061A36 AY232342 ♠
Halothece sp. MPI96P605
GN2013A7(1) AY232320 ♠
GN821A45(50) AY232313 ♣
GN812A20 AY232311 ♣
GN2013A2 AY232319 ♠
Marine stromatolite AF227945
GN2084A08 AY232322 ♦
Xenococcus sp. ATCC 29373 U73135
GN2127A09(1) AY232329 ♦
GN2084A19 AY450628 ♦
Myxosarcina sp. ATCC 29377 U73133
GN1062A41 AY232361 ♦
Marine stromatolite AF227930
Lyngbya lagerheimii UTEX 1930 L15550
GN1063A01 AY232362 ♦
Marine stromatolite AF227937
Plectonema boryanum IU 594 L15552
Dermocarpa sp. ATCC 29731 U73134
GN803A10(7) AY232309 ♣
GN2133A11(6) AY232331 ♦
GN821A16 AY232404 ♣
GN1063A08 AY232364 ♦
Anabaena variabilis ATCC 29413 U49859
GN2133A02(3) AY232330 ♦
Marine mat AY040523
North Carolina estuary UCU73137
GN1064A17 AY232383 ♠
GN2008A01 AY232314 ♠
Fischerella sp. UTEX 1931
Chlorogloeopsis sp. CCAP 1411/1 U73138
Tolypothrix PCC 7101
Anabaena sp. I1 AF124378
GN2008A02 AY232315 ♠
GN1063A12 AY232365 ♦
Mono Lake Ctenocladus aggregate 4 AY221804
GN2127A02(2) AY232327 ♦
Phormidium sp. ATCC 29409 U73132
Neuse river AF035513
Phormidium sp. AD1 AF227927
Pacific Ocean AF059636
Gloeothece sp. L15554
GN1063A15 AY232366 ♦
GN2133A17(3) AY232332 ♦
Neuse river AF035523
Synechocystis sp. WH 8501 AF300829
Stromatolite HBC6C3 AF227934
Pacific Ocean AF299424
GN1064A53 AY232398 ♠
GN803A18(3) AY232310 ♣
Methylomonas sp. LW13 AF378715
Methylomonas methanica AF378717
Marichromatium purpuratum Strain BN5500 AF059649
Marine sediment AF374339
GN1064A23 AY232387 ♠
Neuse river AF035522
GN821A13 AY232402 ♣
GN1064A02 AY232381 ♠
Klebsiella pneumoniae X13303
Vibrio cincinnatiensis AF134809
Vibrio diazotrophicus ATCC 33466 U23650
Pseudomonas stutzeri A15 AJ297529
Azotobacter vinelandii M20568
Azomonas agilis ATCC 7494 AF216883
Rhodospirillum rubrum M33774
Rhodobacter capsulatus X63352
Azospirillum brasilense ATCC 29145 M64344
Sinorhizobium meliloti NC_003037
Mesorhizobium loti NC_002678
Methylosinus sp. LW3 AF378720
Methylocella sp. BL2 AJ491850
Beijerinckia indica ATCC 21423 AF296351
Rhizobium sp. ORS571 M16710
GN1064A40 AY232396 ♠
Spartina alterniflora associated AF216930
Desulfovibrio vulgaris AY040514
0.1 substitutions/site

Bebout, unpublished data). This mat seems to be at or near steady state with respect to growth and decomposition (12) and therefore probably has a low external N requirement (5). The GHM mat had a maximum rate of 40 μmol of C_2H_4 m^{-2} h^{-1}. The reason for the increase in rates in the GHM mat (26) is unclear but may reflect seasonal changes of N dynamics within the mat.

The LG mat had the second highest rates of acetylene reduction. These rates of acetylene reduction, although low relative to rates measured previously in this mat (5; B. M. Bebout, unpublished data), suggest that this mat has a high requirement for fixed N. The LG mat is from an intertidal region, where it is periodically flooded and desiccated. This pattern of alternating wet and dry, as well as displacement of the mat caused by tides, forces the LG mat to be in a state of perpetual growth, in which its demand for fixed N is high (5).

RT-PCR of all three mat samples showed that *nifH* transcripts were present during the night (Fig. 3; data from GNM and GHM mats not shown). Results from negative controls (RNase-treated samples and PCRs without reverse transcriptase) showed that the RT-PCR amplification product was derived from mRNA in the sample and not from contaminating genomic DNA.

nifH gene expression was assayed (Fig. 3) at night, when nitrogenase activity was maximum (18:00 to 2:00 h). Nighttime, or dark-phase, N_2 fixation is typical for most non-heterocyst-forming cyanobacteria (10, 21), such as those found in these mats. Dark-period N_2 fixation could also be a strategy employed by other bacteria to escape inactivation of their nitrogen-fixing apparatus due to high oxygen concentrations. In any event, the *nifH* genes expressed at night are likely to be responsible for the observed acetylene reduction activity. This activity could be regulated by a circadian rhythm, at least for the cyanobacteria, which has previously been demonstrated in several nonheterocystous cyanobacterial species (8, 11, 19).

Cyanobacterial transcripts were the most numerous *nifH* phylotypes in RT-PCR clone libraries in all three mats at night (Table 1 and Fig. 4 and 5). N_2 fixation is an energetically expensive process, as it requires 16 ATP molecules as well as eight reducing equivalents to reduce one molecule of N_2 (32). Cyanobacteria are typically (nondesiccated conditions) not limited by energy in mat environments and thus can probably easily supply the energy and reductant necessary to drive N_2 fixation. Experiments have previously shown that nighttime N_2 fixation is substantially reduced when the activities of photoautotrophs are inhibited during the day by a lack of light or addition of the photosystem II inhibitor DCMU (4, 31). These results suggested the involvement of cyanobacteria in N_2 fixation and indirectly hinted at the type of cyanobacteria involved in this process.

Cyanobacterial *nifH* sequences 97 to 100% identical to sequences from the unicellular halotolerant cyanobacterium *Halothece* sp. strain MPI96P605 were found in the GHM and GNM mats (Table 1 and Fig. 4). The genus *Halothece* is part of a cluster (16S rRNA) of extremely halotolerant cyanobacteria, which are ubiquitous in hypersaline microbial communities (15, 25). Sequences 93 to 97% identical to sequences from other species of unicellular cyanobacteria, such as *Myxosarcina* and *Synechocystis* were found only in the LG mat. It is interesting that unicellular cyanobacteria have been implicated in N_2 fixation in these mats, as they have been detected in numerous environments where N_2 fixation has been suspected (37, 47, 49). However, they have not received attention as major diazotrophs in mats. Recently, unicellular cyanobacteria have been found to be potentially important diazotrophs in the open ocean (51). The results of this study implicate unicellular cyanobacteria in N_2 fixation in mat environments as well.

Phylotypes 95 to 97% identical to that of the second nitrogenase of *Anabaena variabilis* strain ATCC 29413 (41) were also detected in the GNM and LG mats. The second *Anabaena variabilis* nitrogenase is one of three possessed by this organism and is only expressed under anaerobic conditions in vegetative cells (40, 41). *Anabaena variabilis* is a heterocystous cyanobacterium that can fix N_2 during the day in heterocysts (aerobic) or at night in vegetative cells (anaerobic). It is unclear how widely distributed the vegetative nitrogenase is among genera and morphotypes of cyanobacteria and whether it might be evolutionarily closely related to *nifH* genes in nonheterocystous cyanobacteria. Thus, the phylotypes found in the mats could be from heterocystous cyanobacteria very closely related to *A variabilis* or to uncharacterized nonheterocystous cyanobacteria that contain a nitrogenase that is of the second *Anabaena variabilis* type. Heterocysts, which are very visually distinctive, have not been observed directly at the microscope level. We also detected *Phormidium* sp. phylotypes (98 to 100% identical) in the LG and GNM mats, as well as *Plectonema boryanum* phylotypes (98% identical) in the LG and GHM mats by RT-PCR. These genera of cyanobacteria are filamentous nonheterocystous, and the finding of these sequences is consistent with the observation of filamentous nonheterocystous species in the mats.

In addition to cyanobacterial *nifH* phylotypes, phylotypes relating to gamma- and beta-proteobacteria (*nifH* does not adequately distinguish between gamma and beta lineages), and alpha-proteobacteria were found in the GHM and GNM mats. Phylotypes 86 to 88% identical to the *Azotobacter vinelandii nifH* sequence were detected in the GHM mats. This degree of identity is not high enough to assign those sequences to the gamma-proteobacterial lineage. However, these sequences group strongly within the gamma- and beta-proteobacterial

FIG. 4. Phylogenetic tree of cluster 1 of *nifH*, showing the placement of representative mat sequences. Representative sequences were unique sequences from each mat or one sequence chosen to represent sequences from the same mat that showed 98% or greater amino acid sequence identity. Numbers in parentheses represent the number of sequences that were 98% or more identical to the representative sequence. Symbols indicate sequences recovered from genomic DNA (26) and mRNA from the *Microcoleus* (GHM) mat in July 2001(♣); genomic DNA from the *Microcoleus* mat (GNM) in June 2001 (26) and mRNA from October 2001 (♠); and genomic DNA from the *Lyngbya* mat (LG) in June 2001 (26) and mRNA from October 2001 (♦). Bold type indicates sequences that were recovered from mRNA in this study. Bootstrap values of greater than 49 out of 100 replicates are shown at the nodes.

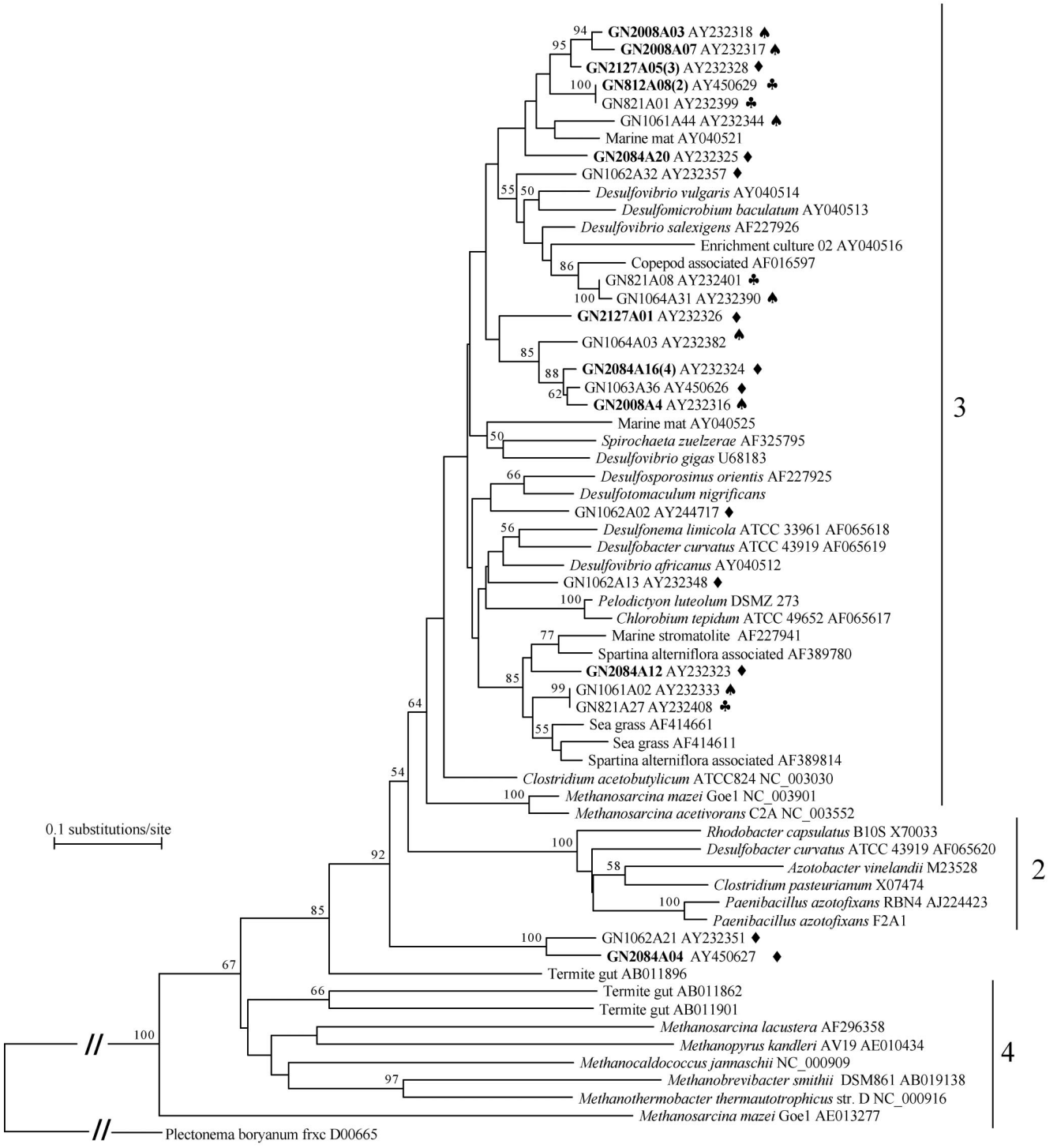

FIG. 5. Phylogenetic tree of *nifH* clusters 2, 3, and 4, showing the placement of recovered representative sequences. Representative sequences were unique sequences from each mat or one sequence chosen to represent sequences from the same mat that showed 98% or greater amino acid sequence identity. Numbers in parentheses represent the number of sequences that were 98% or greater identical to the representative sequence. Symbols indicate sequences recovered from genomic DNA (26) and mRNA from the *Microcoleus* (GHM) mat in July 2001(♣); genomic DNA from the *Microcoleus* mat (GNM) in June 2001 (26) and mRNA from October 2001 (♠); and genomic DNA from the *Lyngbya* mat (LG) in June 2001 (26) and mRNA from October 2001 (♦). Bold type indicates sequences that were recovered from mRNA in this study. Bootstrap values of greater than 49 out of 100 replicates are shown at the nodes.

clade when data sets of *nifH* sequences larger than those used in the analysis presented in Fig. 4 are used, indicating that these sequences are likely to be from gamma- or beta-proteobacteria.

Cluster 3 *nifH* sequences (Table 1 and Fig. 5) were obtained by RT-PCR from all three cyanobacterial mats. This cluster of *nifH* represents several lineages of bacteria, including many anaerobes such as clostridia, sulfate reducers, methanogens, and green sulfur bacteria. None of the recovered sequences were very similar (83 to 89% identity) to any previously recovered isolates. The sequences were most identical (91 to 100% identity) to sequences obtained from identical microbial mats (26) or other habitats that contain anaerobic environments, such as sea grass bed communities (1). The identity of the organisms containing these *nifH* sequences is unclear. The sequences from isolates that were closest to any of the recovered sequences were from sulfate-reducing bacteria. Sequences very similar to those of sulfate-reducing bacteria have also been recovered from cyanobacterial mats in previous studies (26, 37). Recent work by Steppe et al. (36) suggested that *nifH* genes are expressed in sulfate-reducing bacteria and that N_2 fixation in cyanobacterial mats does involve sulfate-reducing bacteria. However, it is also possible that the cluster 3 sequences recovered in this study correspond to multiple and as yet uncharacterized groups of bacteria that are not sulfate reducers.

The sequence GN2084A04 represents a novel and at present uncharacterized *nifH*. Since this sequence could not be ascribed to any particular nitrogenase cluster, it is unclear whether it even functions in N_2 fixation. *nifH* sequences similar to this sequence (91%) have been recovered from similar microbial mats (26). It seems that this sequence does have a function in the environment, since it is actively expressed. This sequence includes a transcription stop codon in its cDNA sequence. Whether or not this stop codon is a result of nucleotide misincorporation during RT-PCR or a natural occurrence is unclear.

Over half of the sequence types recovered in this study were not found in *nifH* PCR clone libraries generated from genomic DNA on similar and identical mats from Guerrero Negro (26). Furthermore, in that study the most abundant sequences recovered were those belonging to cluster 3 organisms (63%), followed by cyanobacteria, which were almost a third less abundant (23%). In this study, cyanobacterial sequences were by far the most abundant sequences (78%), followed by those in cluster 3 (16%). These results suggest that less abundant members of the diazotroph community may have larger roles in N_2 fixation than can be inferred from the abundance of their genes in a DNA-based clone library. However, these differences could also be a result of sampling, environmental, or extraction efficiency differences (26). This was most notable in the LG and GNM mats when these mats were not sampled for DNA and RNA simultaneously. Regardless of the reason for these differences, this study highlights the need to examine expression to determine which organisms are active in N_2 fixation.

The patterns of acetylene reduction and the expression of nighttime *nifH* transcripts indicate that N_2 fixation in these three cyanobacterial mats occurs on a diurnal pattern. Furthermore, the results of this study demonstrate that several groups of cyanobacteria, including unicellular and filamentous types, heterotrophic bacteria, and as yet uncharacterized groups of bacteria express *nifH* in cyanobacterial mats. The *nifH* phylotypes recovered in this study were very similar among the three mats studied, indicating that the same or similar organisms may be involved in N_2 fixation in all three mats. In addition, several of the phylotypes recovered from this study clustered with phylotypes from different environments, such as Mono Lake, a North Carolina estuary (the Neuse River), the Pacific Ocean, and other microbial mats, which suggests that similar organisms may be involved in N_2 fixation in other environments as well. The *nifH* phylotypes identified will provide targets for studies aimed at quantifying the role of diazotrophs in mats and other environments by approaches such as quantitative PCR and RT-PCR as well as DNA and cDNA arrays.

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